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Effect of dietary supplementation of natural immunostimulants on the non-specific immune response of the grey mullet, Mugil cephalus

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Abstract: The purpose of this study was to determine the effect of chitosan scheduled on the non-specific immune mechanisms and disease resistance in Mugil Cephalus. Fish were fed with different doses of chitosan. The non-specific immune mechanisms were assessed in terms of growth parameter, counting of white blood cells (lymphocytes), Red blood cells (erythrocyte), Hematocrit, respiratory burst activity and cumulative survival rate. In our present study was (chitosan 2%) shows a significantly increased growth rate, WBC, RBC, Hematocrit (%), respiratory burst activity and cumulative survival rate compared with another experimental diet. Statistical analysis also proved the cumulative survival rate after 70 days of the challenge test, the experimental group showed 73% survival rate and in the control group, 100% survival was observed. This preliminary study indicates that chitosan could be used to promote the health status of fish in intensive fish aquaculture.

Keywords: Mugil Cephalus, Aeromonas hydrophila, Rice bran, Fish meal, Soybean,

1. INTRODUCTION

Fish and Fishery products have high economic activity all over the world. Fishes are one of the main food components for humans for their high nutritional value [1]**.** *Mugil cephalus,* commonly known as striped mullet or sea mullet is a prevalent fish species. This species is found in tropical waters throughout the world. Sea mullet is an object of the commercial fishery and is not considered a threatened or endangered fish species. The mullet caught on ocean beaches is mostly spawning run fish, and their catches have increased and are considered a highly popular delicatessen fish product. Mullet is successfully cultivated in several countries because of its delicious products [2]. *Mugil cephalus* is one of the most economically important fish that grows in freshwater, brackish, and marine water. Mullet products such as roe, testes, and stomach and delicious taste due to which it has high economic value [3] [4]. Mullets are the most popular species cultivated in Hong Kong due to their reasonable market value [5].

Immunostimulant increases resistance to infectious disease by enhancing non-specific defense mechanisms and not by improving specific immune responses. Hence there is no memory component, and the answer is likely to be of short duration. The use of these

immunostimulants is an effective means of enhancing the immunocompetency and disease resistance of fish. Research on fish immunostimulants is developing and many agents are currently in use in the aquaculture industry [6]. According to Sakai, depending on the sources from which they are derived, immunostimulants can be divided into several groups bacterial, algae-derived, animal-derived, nutritional factors as an immunostimulant, and hormones/ cytokines [7]. A stressor, chemical, drug, or action that boosts the innate or non-specific immune response by interacting directly with cells of the system activating them is known as an immunostimulant. Immunostimulants can be grouped as chemical agents, bacterial preparations, polysaccharides, animal or plant extracts, nutritional factors, and cytokines [8]. Immunostimulants quickly activate non-specific defence mechanisms to protect the fish against pathogens [9]. In aquaculture, the mode of application of immunostimulant is essential along with its dosage, cost of production, level of protection and stress of the fish.

The use of vaccine/antigens directed against a few specific pathogens, whereas immunomodulation directed against recognition of conserved microbial structures by immunostimulant acting on the non-specific defense mechanism, can form an alternative therapy. Chitosan is used as an immunostimulant in aquaculture to protect salmonids and carps against bacterial disease. Chitosan is a linear homopolymer of β-(1,4)-2 amino deoxy - D-glucose. It is prepared by the alkaline deacetylation of chitin, a natural substance obtained from craps shell or any crustacean shell. Anderson and Swicki administered chitosan to brook trout *Salvelinus fontinalis* by injection and immersion. They found that a high production level occurred 1,2,3 day afterward, but production was significantly reduced by day [10]. The present study attempted to monitor the immunostimulant non-specific immune response and growth of *Mugil cephalus* challenging with bacterial pathogen using chitosan as the immunostimulant mixed with feed.

2. MATERIALS AND METHODS

The healthy mullet (*M. cephalus*) fishes were collected from velar estuary (latitude 11º29´ N and longitude 79º46´ E), Parangipettai, southeast of Tamil Nadu, India. Altogether the fishes stage was transferred to the laboratory at the wet condition and supplied randomly in triplicate groups in 300 L rounded plastic tank (fiber reinforce plastic) formfitting with a nonstop flow-through system.

Isolation of bacterial pathogen

A.hydrophila a virulent fish pathogenic strain, was received in Tryptose agar slant, (TSA) from Annamalai university, medical college Annamalainagar Tamil nadu. *A.hydrophila* was sub culture and maintained at 4'C in fish immunology lab, at CAS in marine biology faculty of marine sciences, Annamalai university. A stock culture in Tryptose soya broth (TSB) (Hi-media, Mumbai) was maintained at -40'C with 0.85% Nacl(w/v) and 20% (v/v) glycerol to provide stable inoculum throughout the study period followed by Chabot and Thunne [11].

Experimental feed

Feed ingredient viz, rice born, soybean, fish and wheat meal gained, screened, and evaluated according to usual method. The diet included 2.10% crude lipid, 29.33% crude protein 18.9%, ash and 9.0%humidity. Altogether ingredients were correctly weighed giving to their inclusion rates and ground in an electric grinder separately, carefully mixed and enough water was added then, chitosan was added and mixed to the preparation of the bread in all bulks

with minerals and vitamins. The supporting four diets were prepared in an array to study Diet control, without chitosan and T1, T2 and T3 had the same ingredients as (sigma, USA) supplemented at a level of 1%, 2% and 5% respectively of the diet. Twice daily for a cycle of 70 days experimental standard was fed into all treatments.[12] The frequency of feeding with 5% of the body weight. The growth rate of the fish is summarized in (Table. 1) [13].

Experimental design

Once adaptation of the fish was divided into four treatments of 10 fishes for each group. Group 1 obliged as control and standard diet D1 during the Study Group 2 (Tank 1), Group 3 (Tank 2) and Group 4 (Tank 3) standard diet D2 (1% chitosan), diet D3(2% chitosan), and diet D4 (5% chitosan) separately. The whole experimental season was 70 days at 0day was collected from blood altogether experimental groups the first infection with *A.hydrophila* was specified scheduled the 30th days and the next infection was given on 58th day blood sample were collected from day 30 and 58 in all the experimental groups to study the immunostimulatory consequence of chitosan against *A.hydrophila* infection [15].

Water quality parameters

Water quality parameter analysis was checked frequently in the fish tank, including the temperature, salinity, and pH for each day. It gives the change in the increasing and decreasing of water in temperature, salinity, and pH. In that, the temperature increases by the hot water and decreases by the cold water. In the case of salinity, the salt content increases by saltwater and decreases by freshwater. In pH, it increases by the HCl and decreases by the NaOH.

Blood collection

After completion of the feeding trial of 70 days, the sampling was carried out to analyze the blood parameters, WBC, Hematocrit and respiratory burst activity. Two fish from each duplicate with a total of 20 fish from each treatment were anesthetized with Cifecalm (200 ml L^{-1}). Cifecalm is a herbal anaesthetic formulation contain natural alcoholic extracts of Eugenia caryophyllata and Mentha arvensis (fish immunology lab, at CAS in marine biology faculty of marine sciences, Annamalai university). The blood was collected from the caudal vein with a syringe, which was before rinse through 2.7% EDTA solution. The blood was then transferred immediately near an Eppendorf tube containing a squeeze of EDTA powder, shaken kindly and kept at 4 C**.** The blood was used for the determination of leucocyte count, respiratory burst activity and Hematocrit. On behalf of serum, more than fishs from each treatment were anesthetized and blood was collected not there. Anticoagulant also acceptable to clot for 2 h followed by the collection of straw-colored serum with a micropipette and stored at -20 ^{0}C until use [16].

Blood cell counting

Red blood cell counts (RBCs) and white blood cells (WBCs) were determined with a hemocytometer through Neubauer counting chamber when described by blaxhall [17]. The following method was using to calculate the number of leukocytes per milliliter of the blood sample: Number of cells- (Number of cells counted × dilution/(Area counted × depth of fluied). In this blood smear were given from fresh heparinized blood on microscope slides and discolored with wright-giemga.

Hematocrit
The blood was drawn into heparinised haematocrit pipette up to be 2cm depth using sealant and heat if carefully under the spirit lamp which closed the upper opening. The pipettes were centrifuge for 3 min with a speed of 3000 rpm /min and placed on the analysis tool and reads of the haematocrit rate was expressed % blood cell in total volume of blood [18].

The respiratory burst activity
The respiratory burst activity of the phagocytes was deliberate by Nitroblue tetrazolium assay following the technique of Seacombe's modified by Stasiack and Baumann Fifty microlitres of blood were placed into the wells of 'U'-bottom microtitre plates and incubate at 37 C for 1 h to allow sticking of cells. After that the supernatant was removed with the wells were washing three times with phosphate-buffered saline. Once again washing, 50 ml of 0.2% Nitroblue tetrazolium was additional and incubated for more than 1 hours. The cells were in that case fixed with 100% methanol for 2-3 min and wash three times with 30% methanol. The plates were air-dried and 60 ml of 2 N potassium hydroxide and 70 ml of dimethyl sulphoxide was added to each well. The OD was recorded in an ELISA reader at 540 nm [19].

Statistical analysis
The data were checked from multiple group comparisons the data were analysed by one way ANOVA using a computer program (IBM SPSS Statistics version 22, Armonk, NY, USA), and excel 2010 software, respectively. The comparisons considered significant**, (**$P < 0.005$) [20].

3. RESULT

Hematological parameters are common indicators for recognition of health, stress, and disease conditions in fish. Also, these parameters were evaluated to determine if the potential immunostimulant candidates were harmful to the immune system of fish.

Growth performance
The growth performance of *Mugil cephalus* was studied by dividing into four groups, and three different concentrations of feed, different concentrations of chitosan drugs were mixed with diet feed. The initial value and final value were compared to the fish growth rate. Control group tank, initial weight 15.60 grams and final weight were 18.10 gram using feed with artificial feed, (Tank 1), initial weight 15.20 grams and final weight 17.33 grams using with chitosan drugs 1%, (Tank 2), initial weight 15.10 gram and final weight 23.55 grams using to chitosan drugs 2% and (Tank 3) initial weight 14.50 grams and final weight 16.80 grams using to chitosan drugs 5%. The growth rate value increased to the (Tank 2) diet and was compared with the control diet tank artificial diet group (Table. 1).

Table 1: growth rate and feed utilization of *M.cephalus*

Groups	Control	Chitosan 1%	Chitosan 2%	Chitosan 5%
Initial/W	15.60 ± 0.3	15.20 ± 0.5	15.10 ± 0.6	14.50 ± 0.7

Final/W	18.10 ± 0.5	17.33 ± 0.6	23.55 ± 0.4	16.80 ± 0.6
Initial/L	7.8 ± 0.3	7.7 ± 0.2	7.6 ± 0.1	7.9 ± 0.2
Final/L	15.9 ± 0.7	14.7 ± 0.7	17.6 ± 0.8	14.1 ± 0.7
SGR	3.90 ± 0.03	1.08 ± 0.01	4.33 ± 0.02	3.78 ± 0.016
FCR	8.33 ± 0.2	4.26 ± 0.02	14.08 ± 0.7	4.6 ± 0.01
LGR	10.38 ± 0.3	9.09 ± 0.3	13.15 ± 0.8	7.84 ± 0.1
SR (%)	100 ± 0.2	73.38 ± 0.4	95.56 ± 0.6	65.58 ± 0.8

SGR (%) = (Ln final fish weight – Ln initial fish weight) × 100/time); FCR = feed intake (g)/weight gain (g); LGR (%) = 100 × (ln (final mean body length) − ln (initial mean body length))/culture days; SR (%) = 100 × (final number fish) / (initial number fish).

Water quality parameters

The mean value of temperature varied from 22.05±0.20 to 22.08±0.15 in all the tanks. Salinity ranged from 32.55±0.16 in all the tanks. p^H ranged from 8.10±0.02 to 8.20±0.01 in all the tanks. There was only very minute variation in water quality parameters (Table 2).

Table 2: Water quality parameter of the culture tank

Parameter	Control	Chitosan 1%	Chitosan 2%	Chitosan 5%
Temperature (c)	21.01 ± 0.30	22.05 ± 0.20	22.07 ± 0.50	22.08 ± 0.15
Salinity (ppt)	32.60 ± 1.15	32.80 ± 0.19	32.70 ± 0.18	32.55 ± 0.16
p^H	8.10 ± 0.02	8.13 ± 0.03	8.15 ± 0.04	8.20 ± 0.01

WBC

In the present study fish were divided into four groups in four tanks. In Tank 2 (with 2% of chitosan), there was maximum lymphocyte cells $14.53x10^3$ mm^{-3} at the end of 70 days whereas in control tank 8.90 x 10^3 mm^{-3} lymphocytes cells were found. The lymphocyte cells increased in all groups, but the highest and significant enhancement ($p<0.001$) was observed in Tank 2 with (2% chitosan) (Fig 1).

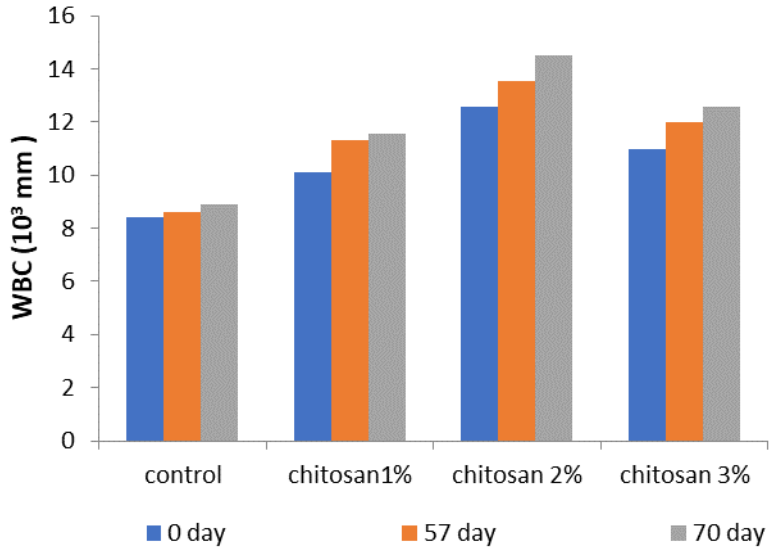

Fig: 1 White blood cell count of *M.cephalus*

RBC

In Tank 2 (with 2% of chitosan), there were maximum erythrocyte cells 2.03x106 mm-3 at the end of 70 days whereas in control tank, there were 1.93x 10^6 mm^{-3} erythrocyte cells were found. The total erythrocyte count was found to be significantly ($p< 0.05$) higher than all the treatment groups camper with control (fig 2).

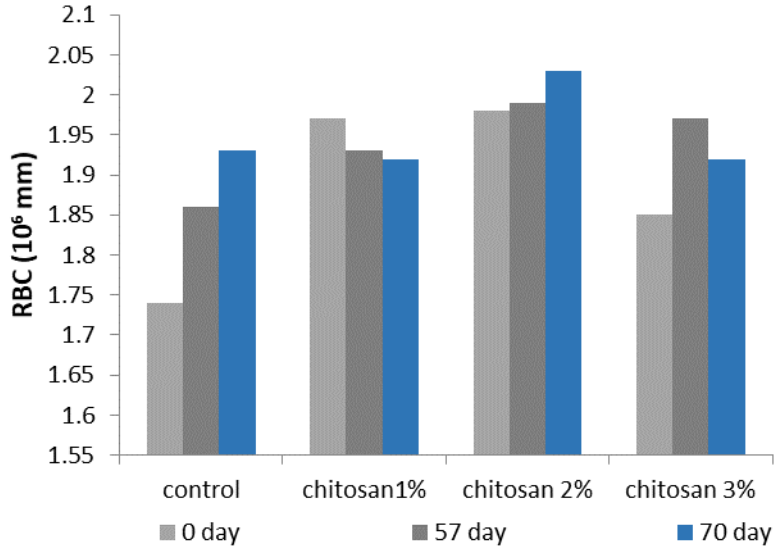

Fig: 2 Red blood cell count of *M.cephalus*

Hematocrit

The control group, Hematocrit (%) was in the normal condition. Due to infection, the hematocrit percentage was decreased in infected groups when compared with control. In this

experimental groups hematocrit percentage was increased in the (Tank 2) and compared with (Tank 1), (Tank 3) and control showed a hematocrit. The statistical analysis showed that Hematocrit was significantly enhanced by all the supplemented diet compared with control group ($p< 0.05$) (fig 3).

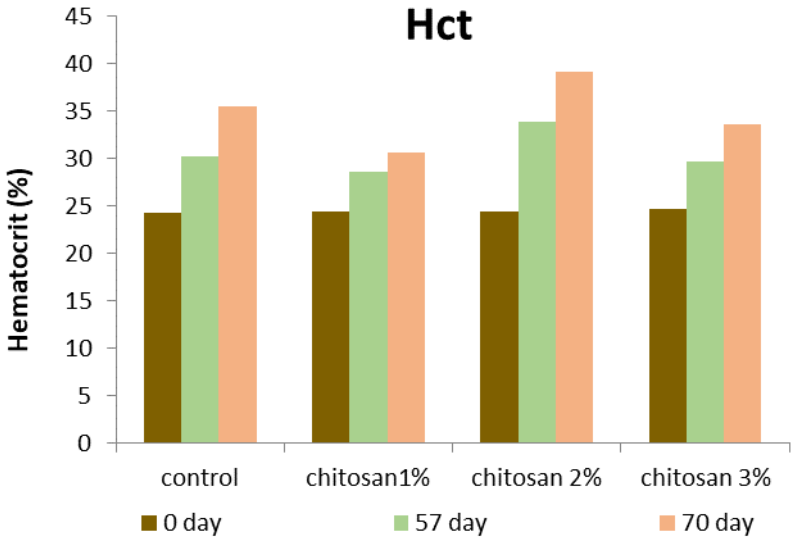

Fig:3 Hematocrit (%) of *M.cephalus*

Respiratory burst activity

Respiratory burst activity (RBA) was presented in (Table 3). The respiratory burst activity of dead kidney macrophages ranged from 0.5 to 0.6 optical density with lowest in control and highest in (Tank 2) chitosan 2% compared with control. The result was significantly increased ($p<0.05$) in a dose-dependent manner in all treatments compared to those in control.

Table:3 Respiratory burst activity of *M.cephalus*

Days	control	chitosan 1%	chitosan 2%	chitosan 5%
0 days	0.5 ± 0.0	0.6 ± 0.01	0.6 ± 0.31	0.6 ± 0.01
57 days	0.6 ± 0.5	0.6 ± 0.30	0.6 ± 0.68	0.6 ± 0.05
70 days	0.6 ± 0.3	0.6 ± 0.63	7.1 ± 0.03	0.6 ± 0.83

Cumulative survival rate

Subsequently feed diet experiment *Mugil Cephalus* remained stimulating through *Aeromonas hydrophila* and detected for the lifetime and after 24 hours stimulating test, experiment group showed 78% lifetime and in control 100% lifetime was detected. After 0 days to 70 days of experimental studies, 73% of lifetime was detected in (Tank1), 95% of lifetime was detected in (Tank 2) and 65% of a lifetime was detected in (Tank 3), control fish 100% of lifetime was detected. The management of immunostimulant about the non-specific immune restriction

and sickness resistance against *Aeromonas hydrophila*in mixed with the diet using chitosan drugs (Fig. 4).

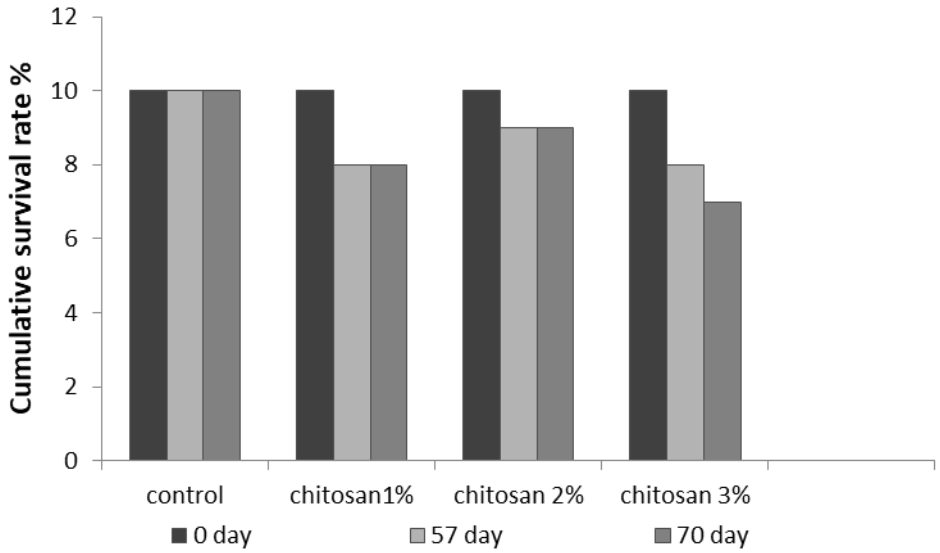

Fig: 4 Cumulative survival rate (%) of *M.cephalus*

4. DISCUSSION

In the present study, *Mugil cephalus* growth rate in terms of length, weight, FCR, LGR, SGR Survival rate was found to be high or maximum in the tank 3 (with 2% of chitosan). These results were like that obtained by Ahmed [22] in *Tilia tomentosa* showed to promote the growth of common carp based on observations of increased WG, SGR and efficiency of feed conversion. These results also agreed with those of previous studies demonstrating that medicinal plants promoted the growth of various aquatic animals [23] [24] [25]. The results also coincided with the results of Seung-Cheol [26] in which WG significantly improved when Japanese flounder (*Paralichthys olivaceus*) were supplemented with an herbal mixture to 500 mg/kg. Yılmaz [27] suggested most probably fat was used for energy, and protein was used for growth in the herbal-supplemented diet. In the present study maximum lymphocyte cells $14.53x10^3$ mm^{-3} were obtained at the end of 70 days in Tank 2 (with 2% of chitosan). Similar results were obtained by Palikova and Navratil [28] where the white blood cells increased in infected or damaged animals. The results also coincided with the results of Kanimozhi [29] where WBC significantly increased in the fish fed with 1 % seaweed extract than control and reached maximum 6.8×10 6 ml-1in 9^{th} days. In Tank 2 (with 2% of chitosan) there was maximum erythrocyte cells $2.03x10^6$ mm^{-3} at the end of 70 days whereas in control tank $1.93x\ 10^6$ mm^{-3} erythrocyte cells were found. In some studies, the positive effects of herbal immunostimulant on the number of RBCs were observed [30] [31] [32]. Their results showed that RBCs increased during the experiment, but there were no differences among the treatments. Brum [33] suggested a negative effect on the number of RBCs and physiological condition of fish is a vital factor to choose a healthy immunostimulant. In the present study significant value $p< 0.04$ was observed for haematocrit [34]. Work results coincides with our results where haematocrit levels in rainbow trout showed no changes after feeding on garlic. Also, Dorucu [35] stated that feeding rainbow trout with 5% Nigella sativa as an immunostimulant increased the level of Haematocrit, however 1 and 2.5% of this plant did

not change the hematocrit level. The respiratory burst activity of dead kidney macrophages was highest in tank 2 (containing chitosan 2%) compared with control. Our results were like the work done by Hajibeglou and Sudagar [36] in which Fish fed on diet with 0.01% D. anethifolia essential oil showed the highest level of respiratory burst activity. Hajibeglou and Sudagar [37] also reported that combination of five herbal extracts increases NBT. Also, garlic as a supplement showed NBT enhancement in some concentrations. Nya and Austin [38] but black cumin seeds showed no effect on NBT assay [39]. The present study was the cumulative survival rate of *M. cephalus* against bacterial pathogen *Aeromonas hydrophila* and detected for the lifetime and after 24 hours stimulating test, experiment group showed 73% lifetime and control 100% lifetime detected. After 0 days to 70 days of experimental studies, 73% of lifetime was detected in Tank1, 95% of lifetime was detected in Tank 2 and 65% of lifetime was detected in Tank 3, control fish 100% of lifetime was detected. According to Dügenci [40] the survival rate of infected fish usually increased after being treated with various immunostimulant vaccines and probiotics. The mortality of *O.niloticus* challenged with *A. hydrophilia* decreased with increased *C.sinenis* level in supplemented diets. Our results were like the results was reported by Abbas and Siddiqui [41]. Where SGR was observed in SW5 (2.08%) diet and SW2 (1.87%). Similar SGR (2.17%) was reported when fish was fed with 42% fishmeal cum soybean meal-based diet for 75 days Luo [42]. reported that SGR varied between 1.1% to 1.5% [39]. In contrast, Millamena reported higher values of SGR ranging between 2.8 to 3.2 Abbas [43] reported that the highest SGR of 2.65% was obtained by the diet containing 40% and Siddiqui and Khan [44] reported that *Heteropneustes fossilis* fed with 40% of protein showed 1.76% of SGR.

5. CONCLUSION

It is concluded that chitosan drugs supplemented diet at the concentration relatively enhanced the non-specific immune response in *M.cephalus* fingerling and remarkably decreased the mortality against *Aeromonas hydrophila*.

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